The periphery of the developing collagen fibril

Quantitative relationships with dermatan sulphate and other surface-associated species

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(Received 30 December 1982/Accepted 15 September 1983)

1. Dermatan sulphate, hydroxyproline and collagen fibril diameters were measured in flexor tendons from chick and calf limbs, from early in embryonic development to maturity. 2. The collagen fibril is viewed as a long thin cylinder. A species X present at the periphery of the cylinder, regularly and specifically arrayed along the fibril, should then satisfy the relationship

$$[X]/[\text{collagen}]r = k$$

where [X] and [collagen] are tissue concentrations of X and collagen, and r is the fibril radius. 3. Throughout the developmental period studied, dermatan sulphate (i.e. X) in chick, calf and rat tendons fits the relationship, implying that it is specifically, regularly and entirely associated with collagen fibrils, thus confirming and extended previous electron histochemistry [Scott & Orford (1981) *Biochem. J.* 197, 213–216]. 4. This approach explains the pattern of change of dermatan sulphate content during development of the tendon. 5. The findings imply that the dermatan sulphate proteoglycan–collagen interaction is evolutionarily highly conserved. 6. The relationship

$$[X]/[\text{collagen}]r = k$$

is used to show that surface concentrations of covalently bound species, such as extension propeptides, can be easily assessed, given the data base described in paragraph 1 above.

It is becoming ever clearer that the surface of the collagen fibril plays many active roles in the organization and function of connective tissues. Proteoglycans and fibronectin *inter alia* associate specifically with certain regions of the fibril, as defined by electron microscopy (Scott, 1980; Furcht *et al.*, 1980; Scott & Orford, 1981). It has been suggested that these interactions help to control the increase in size and degree of cross-linking of developing fibrils (Scott *et al.*, 1981b), as well as tissue calcification at later stages (Scott & Orford, 1981). In view of the importance of these functions throughout the animal world, quantitative and qualitative aspects of fibril surface interactions must be investigated by as many independent methods as possible. Electron microscopy, which in principle is the most direct approach, has been used most, but the results are usually artifacts of staining, embedding and tissue preparation, to be interpreted with appropriate reservations. The

present paper describes an alternative principle, based on parallel biochemical and electron-microscopic measurements, which does not entail staining artifacts, and which gives quantitative data on the stoichiometry of the complexes formed at the surface of the collagen fibril. Some of this work has been preliminarily communicated (Scott *et al.*, 1981a).

The collagen fibril is seen as a very long thin cylinder, of length l , radius r , surface area $2\pi rl$, and total volume $\pi r^2 l$. If a species X is associated with the fibril surface in a regular way, the total amount of X = $k'2\pi rl$. k' is then the quantity of X associated with unit surface area of the cylinder of collagen. Total X is related to the total volume of the cylinder (i.e. the total collagen) by $k'2\pi rl/k''\pi r^2 l$, i.e. by kr^{-1} . In other words, the ratio of total X in a piece of tissue to the total collagen will be greater, the smaller the fibril radii. We can replace 'total X', or 'total collagen per piece of

tissue' with $[X]$ and $[\text{collagen}]$, the concentrations of X and collagen respectively, so that:

$$[X]/[\text{collagen}] = k/r$$

or

$$([X]/[\text{collagen}])r = k$$

In a series of tissue samples, each with different fibril radii, a plot of the ratio $[X]/[\text{collagen}]$ against r^{-1} should be a straight line, or alternatively $([X]/[\text{collagen}])r$, which I term product I , should be a constant, the same for each sample.

Thus by biochemical determinations of $[X]$ and $[\text{collagen}]$ on a series of samples with collagen fibrils of different known radii, the hypothesis that X is regularly associated at specific loci on the collagen fibril can be tested, by the constancy or otherwise of product I . Although initial investigations require considerable effort, particularly in accumulating fibril diameter statistics by electron microscopy, succeeding investigations on the same system are relatively fast, requiring only measurements of the concentrations of X and collagen.

The present paper discusses the distribution at the collagen fibril surface of non-covalently bound dermatan sulphate proteoglycan, and covalently bound N -terminal extension propeptides, in the light of this approach. The results are relevant to previously published speculations on the control of collagen-fibril lateral growth.

Experimental

Materials

Flexor tendons were taken from fore-limbs and hind-limbs of eight foetal calves at various stages during pregnancy, and from mature cows. Foetal crown-rump length, which is related to intra-uterine age (Bogart, 1959), was measured. Flexor tendons taken from chicken (A. S. A. Warren breed) legs at 17, 19, 22, 29, 39, 55, 68 days and 18 months after fertilization were divided into four zones, zone 1 at the muscle junction, zone 2 proximal to the heel, zone 3 at the level of the heel, and zone 2' distal to the heel. Zone 2' was used in the present investigations, as typical tendon.

Methods

Measurements of tendon collagen-fibril diameters, hydroxyproline, dermatan sulphate, chondroitin sulphate and hyaluronate were made by the methods described previously (Scott *et al.*, 1981b). Analyses on chick tendon were on pooled samples, at least 12 tendons being taken for each age during early embryonic development and two to four at later post-hatching stages. Most data points represent the means of two separate experiments. Analyses on bovine tendon were on single samples.

N -Terminal extension propeptides were assayed on collagenase digests of calf tendon by the methods of Timpl and co-workers (Rohde *et al.*, 1976, 1978).

Results

During the development periods studied, the tendon collagen-fibril diameters increased in size from about 30 nm to over 200 nm (Table 1). The size distributions were narrow, except for the older specimens. The standard errors are given in Table 1. Hydroxyproline content per g dry wt. also increased, sharply during the earliest periods, from less than 10 mg/g (Scott *et al.*, 1981b), levelling off before maturity at about 90 mg/g (Table 1). The rise in hydroxyproline content preceded the increase in collagen fibril radii. Chondroitin sulphate and hyaluronate contents/g dry wt. decreased very rapidly during the early periods, to a low plateau extending to maturity (results not shown). Dermatan sulphate contents per g dry wt. increased gradually in the early period, then declined somewhat towards maturity (Table 1). These results resemble those reported on rat tail tendon (Scott *et al.*, 1981b).

For the purposes of the present paper, dermatan sulphate is defined as that sulphated polyanion which is precipitated at 23.5% (v/v) ethanol in the presence of calcium acetate, and reacts in the periodate/Schiff colorimetric assay for L-iduronic acid (see Scott *et al.*, 1981b). Chondroitin 4-sulphate-like polymers, which are precipitated at considerably higher concentrations of ethanol, are thus excluded. The results follow the same trend as an electrophoretic assay for dermatan sulphate, based on mobility in calcium acetate buffer (Scott *et al.*, 1981b, and unpublished work).

The N -terminal-extension-propeptide/solubilized-collagen weight ratios for the tendons from calves of 33, 45, 55 and 77 cm crown-rump length and mature cow were 0.00011, 0.00011, 0.00010, 0.00007 and 0.00002 respectively.

The plot of hydroxyproline/dermatan sulphate ratio against fibril diameter is shown in Fig. 1. The data on rat tail tendon are taken from Scott *et al.* (1981b). The two parameters are very significantly correlated. Kendall's rank correlation coefficient = 0.824, and Z , the standard normal variate = 5.504, with $P < 0.00001$ (Siegel, 1956). The slope of the Pearson regression line (Fig. 1) is 1.180 (S.E.M. ± 0.064), $r = 0.97$. The data were further tested to see whether a quadratic function fitted the data better than a straight line. The quadratic term was insignificant, however ($P = 0.15$). I am indebted to Dr. A. Gibbs for this calculation. All eight points from chick tendon zone 2' lay close to, or on, the best-fit line. Only one of the six from rat

Table 1. Fibril diameters and the contents of hydroxyproline and dermatan sulphate in bovine and chicken limb flexor digitorum tendons

$$\text{Product } I = \left(\frac{[\text{dermatan sulphate}]}{[\text{hydroxyproline}]} \right) \times \text{radius of fibril.}$$

Bovine flexor tendon				
Foetal crown-rump length (cm)	Fibril diameter (mean $\pm$ S.E.M.) (nm)	Hydroxyproline (mg/g dry wt.)	Dermatan sulphate (mg/g dry wt.)	Product <i>I</i>
12.5	33.0 $\pm$ 0.5	36.5	1.56	0.70
25.0	32.6 $\pm$ 0.5	56	3.84	1.12
32.0	38.9 $\pm$ 0.5	50	3.88	1.51
36.0	38.3 $\pm$ 0.6	66.6	4.18	1.20
47.5	44.9 $\pm$ 0.6	67	4.2	1.41
54.5	43.8 $\pm$ 0.7	70	3.47	1.09
74.5	66.0 $\pm$ 1.3	84	3.23	1.26
89.0	65.2 $\pm$ 1.1	88	3.35	1.24
Adult	140. $\pm$ 4.4	94.5	1.35	1.0
Chicken flexor tendon				
Days after fertilization	Zone-2' fibril diameter (mean $\pm$ S.E.M.) (nm)	Hydroxyproline (mg/g dry wt.)	Dermatan sulphate (mg/g dry wt.)	Product <i>I</i>
17	46.4 $\pm$ 0.3	43.5	1.31	0.70
19	47.4 $\pm$ 0.3	55.4	2.26	0.97
22	48.6 $\pm$ 0.4	67.6	2.2	0.79
29	70.5 $\pm$ 0.9	67.5	2.26	1.18
39	86.1 $\pm$ 1.6	74.7	1.34	0.77
55	133.0 $\pm$ 2.7	71.0	1.2	1.12
68	140.4 $\pm$ 3.3	80.6	0.98	0.85
Mature	210.0 $\pm$ 5.4	86.0	0.79	0.96

tail tendon deviated significantly, and the nine bovine tendon points lay near the line. Only three of the 23 values of product *I* from rat tail, chick zone-2' and bovine tendons fall outside the range 0.7–1.3 (average 0.99).

Analyses carried out on tendon zone 2, proximal to the heel, gave values of product *I* that ranged from 0.55 to 1.51 (average 0.88). Because of the much smaller size of this part of the tendon, compared with zone 2', the analyses were single, on much smaller amounts of dermatan sulphate, and therefore intrinsically less precise. For this reason they are not plotted with the other data in Fig. 1.

By contrast, the value of product *I* for chondroitin sulphate and hyaluronate varied by more than 10-fold (Scott *et al.*, 1981a, and unpublished work), the values for thin fibrils being much higher than those for thick fibrils.

Discussion

The developing tendon is a system that offers prospects of testing the proposed relationships on a range of fibril thicknesses varying by more than 10-fold (Scott *et al.*, 1981a,b). Fibril diameters and collagen (i.e. hydroxyproline) contents have been measured throughout the age range, before and

after parturition or hatching, in the rat tail, chick leg and bovine limb flexor tendons (Scott *et al.*, 1981b; Table 1). This data base provides the foundation on which tests of the possibility of regular fibril surface association by any compound or group can be made rapidly and conveniently, by biochemical analysis.

Dermatan sulphate

The very highly significant correlation demonstrated in Fig. 1 and the Results section between fibril diameters and the hydroxyproline/dermatan sulphate ratio implies that dermatan sulphate is regularly, specifically and entirely associated with tendon collagen fibrils throughout most of development, in different species. This result confirms and extends the electron-histochemical picture, in which specific staining showed proteoglycan to be regularly present at the 'd' band in the gap zone of rat tail tendon collagen fibrils (Scott & Orford, 1981). In older animals at least, biochemical analyses and hyaluronidase digestions suggested strongly that the proteoglycan was dermatan sulphate-rich. Examination of the plot in Fig. 1 shows that the data for rat tail, bovine flexor and chick zone-2' tendons cluster closely to the best-fit line, implying that the relationship is indeed linear,

Fig. 1. Plot of hydroxyproline/dermatan sulphate ratio against collagen-fibril diameter for bovine flexor tendon (○), rat tail tendon (●) and chicken flexor tendon zone 2' (▲).

See the text for details. The data for rat tail tendon are from Scott *et al.* (1981b). The best-fit line is a Pearson regression line, slope 1.18, intercept - 3.9, $r = 0.967$.

as required by the expression for product *I*. The bovine data do not cover as wide a range as those from chick and rat, and by themselves do not constitute a complete test of the model, since there is only a single point in the region of larger fibril radii, but the very close fit of the points to the common line strongly supports the main conclusions. The Kendall ranking procedure does not prove the linearity of the relationship, but the present data, which cover a 10-fold range in each variable, present no clear alternative.

The spread in product *I* values for chondroitin sulphate and hyaluronate in these samples is much greater (more than 10-fold), with the values for thinner fibrils being always much higher than those for thicker fibrils (Scott *et al.*, 1981a, and unpublished work). This implies that the relationships of chondroitin sulphate and hyaluronate with the periphery of the collagen fibril are not constant throughout development, in contrast with those of dermatan sulphate.

Since the points from three different species cluster about the same line, it is implied that there is little species difference in the constant of proportionality, k' , between dermatan sulphate proteoglycan and collagen-fibril surface area. This suggests that this interaction will be found to be evolutionarily highly conserved. This would be expected, if speculations on the effect on calcification and fibril cross-linking of the placement of dermatan sulphate proteoglycan at the gap zone (Scott & Orford, 1981) are borne out.

The approach in the present paper provides an explanation for the observed pattern of a rise and then a fall in the content of dermatan sulphate per

g of tissue during early development of tendon, as the outcome of opposing trends. The rise is the consequence of an increasing number of fibrils/g of tissue (as deduced from the considerable increase in hydroxyproline content while the fibril radii remain relatively constant), resulting in more fibril surface area per g of tissue, and hence more dermatan sulphate. The fall follows upon a decrease in fibril surface area, as more of the collagen is to be found in thicker and thicker fibrils. The rise-fall pattern in dermatan sulphate content has been observed in other tissues (Nakamura & Nagai, 1980), where a set of events similar to those occurring in developing tendon may be suspected.

Two simple alternative models can be constructed. The first, in which dermatan sulphate is distributed regularly and solely inside the collagen fibril, would imply that the hydroxyproline/dermatan sulphate ratio was constant, which is clearly not the case (Table 1). The second, that it is distributed regularly throughout the volume outside the fibrils, would imply a steady decrease in tissue dermatan sulphate content, as the content of collagen (hydroxyproline) increases with maturation, and this is also not the case (Table 1). Neither model is compatible with the distribution of dermatan sulphate shown in the electron microscope (Scott, 1980; Scott & Orford, 1981), which supports only the model proposed in the present paper.

It is possible, in principle, to calculate approximately how many molecules of dermatan sulphate per repeating (D') fibril unit correspond to the observed relationship, given the density of the collagen fibril (1.3 g/ml; Noda, 1972) and the hydroxyproline content of collagen. Assuming an M_r value for the dermatan sulphate proteoglycan of about 100000–200000 (by analogy with other tissues; see, e.g., Cöster, 1979; Fuji & Nagai, 1981; Pearson & Gibson, 1982), the number of dermatan proteoglycan molecules associated with the surface of a D unit of a collagen fibril of diameter 50 nm is of the order of 10–20 (Scott *et al.*, 1983).

Covalently attached species

Whereas the above procedures tested the hypothesis that X (dermatan sulphate) was associated regularly with the collagen fibril, where X was not covalently bound to the collagen fibril, they can also be used to test that species covalently bound to tropocollagen, e.g. hydroxylysine glycosides, or aminopropeptides, are present solely at the surface, rather than in the interior, of the fibril. Provided that the surface-associated species X is of known structure, or molecular mass, X/collagen is a simple, directly calculable, weight ratio. Given the lateral dimensions of the collagen molecule

(1.5 nm diameter), it is easy to calculate the number making up the outer annulus of collagen-X molecules in a fibril of given size. Making reasonable assumptions, the total number of collagen molecules in the fibril can then be calculated. From the resulting ratio (no. of collagen-X molecules)/(total no. of collagen molecules) the ratio $[X]/[\text{collagen}]$ for a fibril of given size can be calculated and thence an 'expected' value of product I can be derived. If the observed ratio were higher than expected, X would be present in other places, in addition to the periphery of the fibril. If less, there would be insufficient X to be covalently bound to all the peripheral collagen.

N-Terminal extension propeptides

It is a simple matter to calculate and compare the theoretical values for product I for fibrils of various diameters and containing collagen molecules at different packing densities, since $[X]/[\text{collagen}]$, the weight ratio of aminopropeptide/type I procollagen, is about 0.10, assuming M_r values of about 15000 and 340000 respectively. If all peripheral collagen molecules possessed their propeptides, the penta-fibril (diameter about 4 nm) would give a product I of approx. 0.19.

In moist stretched rat tail tendon, from the crystallographic data of Hulmes & Miller (1979) the number of molecules in a cross-section of a 30 nm-diameter fibril is estimated to be 350 ± 30 (J. Chapman, personal communication). The outer ring of collagen molecules, occupying an annulus of average diameter 29.3 nm, would contain about 60 molecules of diameter 1.5 nm, giving an *N*-terminal-extension-propeptide/total-collagen weight ratio of about $60 \times 0.10/350$, i.e. 0.017. The product I would be 0.26.

Preliminary results, obtained on calf flexor tendons from the foetal calves of 33, 45, 55 and 77 cm crown-rump length, and mature cow, showed fairly constant values of product I of 0.0019, 0.0023, 0.0024, 0.0023 and 0.0014 respectively (Results section and Table 1). On this basis

N-terminal extension propeptides are probably regularly present at the periphery of the collagen fibril, but the ratios are about 100-fold less than the calculated expected value, suggesting that conversion from procollagen is essentially complete, during the developmental period studied.

My thanks are due to Ms. E. W. Hughes, Mr. B. Marcyniuk and Dr. C. R. Orford for technical assistance, and to Dr. Sam Lucas for advice and help with statistics and computation.

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